

Influence of the Nitrogen Source on *Saccharomyces cerevisiae* Anaerobic Growth and Product Formation

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To prevent the loss of raw material in ethanol production by anaerobic yeast cultures, glycerol formation has to be reduced. In theory, this may be done by providing the yeast with amino acids, since the *de novo* cell synthesis of amino acids from glucose and ammonia gives rise to a surplus of NADH, which has to be reoxidized by the formation of glycerol. An industrial strain of *Saccharomyces cerevisiae* was cultivated in batch cultures with different nitrogen sources, i.e., ammonium salt, glutamic acid, and a mixture of amino acids, with 20 g of glucose per liter as the carbon and energy source. The effects of the nitrogen source on metabolite formation, growth, and cell composition were measured. The glycerol yields obtained with glutamic acid (0.17 mol/mol of glucose) or with the mixture of amino acids (0.10 mol/mol) as a nitrogen source were clearly lower than those for ammonium-grown cultures (0.21 mol/mol). In addition, the ethanol yield increased for growth on both glutamic acid (by 9%) and the mixture of amino acids (by 14%). Glutamic acid has a large influence on the formation of products; the production of, for example, α -ketoglutaric acid, succinic acid, and acetic acid, increased compared with their production with the other nitrogen sources. Cultures grown on amino acids have a higher specific growth rate (0.52 h^{-1}) than cultures of both ammonium-grown (0.45 h^{-1}) and glutamic acid-grown (0.33 h^{-1}) cells. Although the product yields differed, similar compositions of the cells were attained. The NADH produced in the amino acid, RNA, and extracellular metabolite syntheses was calculated together with the corresponding glycerol formation. The lower-range values of the theoretically calculated yields of glycerol were in good agreement with the experimental yields, which may indicate that the regulation of metabolism succeeds in the most efficient balancing of the redox potential.

Ethanol production by yeast cultures is an important industry. Important products are beer, wine, distilled potable alcohol, and fuel alcohol, and the last of these will probably become even more important in the future. However, to make ethanol an economically feasible biofuel, the process has to be optimized in terms of yields as well as ethanol production rate (3). The ethanol yield may be improved by repressing the formation of biomass and by-products, among which glycerol is the most important. The reduction of glycerol production may also make the purification process cheaper.

Glycerol plays a role in the osmotic regulation of the cell (1), but under "normal" osmotic conditions the main function for glycerol production under anaerobic conditions is to maintain an intracellular redox balance (21, 22). During the formation of glycerol, the surplus of NADH, originating mainly from the synthesis of amino acids (33), is converted to NAD^+ . In many industrial media, the nitrogen source consists of different substances, such as peptides and free amino acids. In such media the formation of glycerol may be reduced compared with that when, for example, ammonium salt is the only available nitrogen source.

Relatively few studies have investigated the influence of the nitrogen source on yeast growth and product formation. The studies done have mainly been focused on beer fermentation, in which the media contain both ammonium and amino acids. Mäkinen et al. (19) showed that a high concentration of amino acids in the wort increased the fermentation rate and acceler-

ated the growth of the yeast under both aerobic and anaerobic conditions. Wheat mashes were used by Thomas and Ingledew (30) in a study of the effect of amino acids on the fermentation and growth of yeast cells in very-high-gravity fermentations (very high sugar concentrations). They found that mixtures of amino acids (as in yeast extract or Casamino Acids) or a single amino acid (such as glutamic acid) stimulated growth and decreased the fermentation time. Radler and Schütz (24) studied anaerobic glycerol formation as a function of the nature and amount of the nitrogen source and found that some single amino acids (alanine, asparagine, serine, and valine) caused a decreased level of glycerol formation compared with that when a mixture of amino acids was used.

In the present study, the influence of different nitrogen sources on the growth and product formation of an industrial strain of *Saccharomyces cerevisiae* was examined. Three different nitrogen sources were used: ammonium salt, glutamic acid, and a mixture of 20 amino acids representative of an industrial medium based on wheat hydrolysate. Glutamic acid is central in the biosynthesis of amino acids and is therefore an appropriate choice for a single amino acid as the nitrogen source. Anaerobic batch cultivations were monitored on-line by flow microcalorimetry and gas analysis. Samples were also taken for analysis of metabolites and of cell composition. Furthermore, on the basis of synthesis of amino acids, RNA, and extracellular metabolites, theoretical calculations of NADH formation and the corresponding glycerol production were performed.

MATERIALS AND METHODS

Strain and media. An industrial strain of *S. cerevisiae* (supplied by the baker's yeast manufacturer Jästbolaget AB, Rotebro, Sweden) was used. The yeast was

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TABLE 1. Medium composition

Component	Amt/liter
Minerals	
KH ₂ PO ₄	3.51 g
MgSO ₄ · 7H ₂ O.....	0.74 g
Trace metals	
EDTA.....	30.0 mg
CaCl ₂ · 7H ₂ O.....	9.0 mg
ZnSO ₄ · 7H ₂ O.....	9.0 mg
FeSO ₄ · 7H ₂ O.....	6.0 mg
H ₃ BO ₃	2.0 mg
MnCl ₂ · 7H ₂ O.....	1.56 mg
Na ₂ MoO ₄ · 7H ₂ O.....	0.80 mg
CoCl ₂ · 7H ₂ O.....	0.86 mg
CuSO ₄ · 7H ₂ O.....	0.60 mg
KI.....	0.20 mg
Carbon and energy source (D-glucose).....	20 g
Vitamins^a	
D-Biotin.....	0.05 mg
<i>p</i> -Aminobenzoic acid.....	0.2 mg
Nicotinic acid.....	1.0 mg
Calcium pantothenate.....	1.0 mg
Pyridoxine HCl.....	1.0 mg
Thiamine HCl.....	1.0 mg
<i>m</i> -Inositol.....	25.0 mg
Other	
Ergosterol ^b	10.0 mg
Tween 80 ^b	0.42 g
Antifoam ^c	100 µl

^a Prepared as described by Bruinenberg et al. (2).

^b Prepared as described by Verduyn et al. (33).

^c Polypropylene glycol P2000 (Fluka Chemika-Biochemika, Buchs, Switzerland).

maintained on agar plates containing (per liter) 20 g of bacteriological agar (Oxoid Agar no. 1; Unipath LTD, Basingstoke, Hampshire, England), 10 g of yeast extract (Difco Laboratories, Detroit, Mich.), 20 g of peptone (Difco), and 20 g of D-glucose (BDH Laboratory Supplies, Poole, England).

One of the following nitrogen sources was added to the medium at the indicated concentration: ammonium [7.5 g of (NH₄)₂SO₄ per liter], glutamic acid (5.0 g/liter, corresponding to 34.0 mmol of N per liter), or a mixture of 20 amino acids (34.0 mmol of N per liter). The mixture of amino acids was composed of the following (in milligrams per liter): alanine, 181.7; arginine, 241.4; asparagine, 601.9; aspartic acid, 484.2; cysteine, 8.2; glutamine, 12.4; glutamic acid, 360.2; glycine, 84.2; histidine, 36.9; isoleucine, 22.3; leucine, 71.4; lysine, 84.5; methionine, 20.3; phenylalanine, 50.5; proline, 31.3; serine, 71.5; threonine, 36.5; tryptophan, 482.6; tyrosine, 49.3; and valine, 71.7.

The inoculum and experimental cultures were grown in the same defined medium (Table 1), except that ergosterol and Tween 80 were excluded in the inoculum cultures. The inocula (50 ml) were grown aerobically for approximately 36 h at 30°C in 300-ml shake flasks. The same nitrogen source as in the subsequent anaerobic cultivation was used.

The mineral components, including trace metals and antifoam, and glucose were autoclaved separately at 121°C for 15 min. The ergosterol-Tween 80 solution was heated to 100°C for 5 to 10 min in a water bath before addition to the medium, whereas the solutions of vitamins, glutamic acid, and the mixture of amino acids were filter sterilized.

Bioreactor system. Cultivations were carried out in a Belach bioreactor system (Belach Bioteknik AB, Stockholm, Sweden) with a working volume of 3.0 liters (seven experiments) or 2.0 liters (two experiments) (no differences in the results due to different cultivation volumes were observed). The bioreactor was equipped with a condenser and sterilizable electrodes for measurements of pH, temperature, and dissolved oxygen. Carbon dioxide was analyzed continuously with an acoustic gas analyzer (carbon dioxide and oxygen monitor, type 1308; Brüel and Kjær, Naerum, Denmark) described by Christensen et al. (4). The rate of heat produced was measured with a flow microcalorimeter (thermal activity monitor, model TAM 2277; ThermoMetric AB, Järfälla, Sweden). This microcalorimetric setup has been described previously (16), but in this study no air was added to the flow, resulting in an effective volume of the flowthrough cell of 0.52 ml. The agitation rate was controlled at 500 rpm, the temperature was controlled at 30°C, and the pH was controlled at 5.0 by addition of 1 M NaOH. The dilution caused by base addition was corrected for in the metabolite analyses. In order to maintain anaerobic conditions, the fermentor was flushed with pure nitrogen (<1 ppm O₂) (N48; Air Liquide, Malmö, Sweden) at a controlled flow rate of 0.25 vol/vol/min at normal temperature and pressure with a mass flow controller

(Bronkhorst High-Tech BV, Ruurlo, The Netherlands). Evaporated ethanol was determined in separate experiments, using the same setup and conditions as for the cultivations but with water-ethanol solutions. It was found that 0.10 g/liter/h was lost for an ethanol concentration of 9.5 g/liter in the solution. In further calculations, the losses were assumed to be proportional to the ethanol concentration in the medium.

Batch cultivation procedures. The bioreactor containing the mineral part of the medium was autoclaved at 121°C for 15 min with an extra pressure support at the end of the sterilization cycle. Glucose, ergosterol-Tween 80, vitamins, and the nitrogen source when glutamic acid or the mixture of amino acids was used as the nitrogen source were added after cooling of the bioreactor. The fermentor was inoculated with 50 ml of inoculum culture, bringing the volume to the total working volume. The initial cell concentrations were 0.05, 0.10, and 0.12 g (dry weight) per liter for ammonium, glutamic acid, and amino acid mixture cultures, respectively.

Analytical methods. (i) Liquid components. For analysis of liquid components, samples were centrifuged (15,000 × g) for 5 min and the cell-free supernatants were stored at -20°C until analyzed.

Metabolites were measured with a liquid chromatograph (Waters Chromatography Division, Millipore Corporation, Milford, Mass.) equipped with a refractive index detector and a UV detector (210 nm). Glucose, glycerol, ethanol, pyruvic acid, succinic acid, acetic acid, α-ketoglutaric acid, fumaric acid, malic acid, *cis*-aconitic acid, and 2,3-butanediol were separated on a polymeric ion-exchange column (Shodex SH 1011; Showa Denko K.K., Tokyo, Japan) at 55°C with 5 mM H₂SO₄ as the eluent (1.0 ml/min). Citric acid and isocitric acid were analyzed with enzymatic kits (Boehringer, Mannheim, Germany).

(ii) Determination of nitrogen-containing compounds. Ammonium and glutamic acid were analyzed with enzymatic kits (Boehringer). A free amino acid nitrogen (FAN) method was also used for cultures containing a mixture of amino acids or glutamic acid for measurements of the total amino acid nitrogen. The FAN method is a ninhydrin-staining method from the European Brewery Convention (7). Glycine and aspartic acid solutions were used as standards. Because of different absorptivities of different amino acids, the FAN method underestimates the nitrogen content of a sample containing a mixture of amino acids. A correction factor of 1.6 was calculated from the measured FAN value (grams of nitrogen per liter) and the independently measured amino acid concentrations. This factor was subsequently used in the FAN determinations of total amino acid nitrogen. Individual amino acid concentrations were measured by ion-exchange chromatography (20, 28, 29) with detection of the ninhydrin-stained amino acids at 440 and 570 nm. These analyses were done at the Central Laboratory of Clinical Chemistry (Sahlgrenska Hospital, Göteborg, Sweden), except for aspartic acid and asparagine, which were analyzed as follows at the Amino Acid Analysis Laboratory (Department of Biochemistry, University of Uppsala, Uppsala, Sweden). To 1 volume of sample were added 2 volumes of lithium citrate-buffered sulfoxylic acid (pH 1.8) (approximately 3% in system buffer [0.15 M lithium, 0.07 M citrate]) and an appropriate amount of norleucine (750 to 1,250 nmol/ml of sample). The resulting solutions were chilled on ice for 60 min to precipitate any protein present. Following centrifugation at 10,000 × g, 50-µl aliquots were analyzed with a Biotronik LC 5001 amino acid analyzer, using the extended physiological system with lithium citrate buffers and ninhydrin detection. Data were collected with a Shimadzu CR2AX integrator, and the results were normalized on the basis of the recovery of the added internal standard (norleucine).

(iii) Biomass determination. The biomass concentration was determined by turbidity measurements at 610 nm, correlated to dry weight from duplicate samples (5 ml, taken in the middle and at the end of cultivation) which were centrifuged (5,000 × g, 10 min), washed twice with distilled water, and dried for at least 24 h at 110°C. The specific growth rate was calculated from the biomass measurements, as well as from the heat measurements, since during anaerobic exponential growth, the rate of heat production is directly correlated to the specific growth rate.

(iv) Total protein content of cells. Total protein content was determined with samples of freeze-dried cells (prepared as described previously [9]), resuspended in 3 ml of 1 M NaOH, by a modified biuret method (34). The A₅₅₅ was measured, and bovine serum albumin (Amersham Life Science, Little Chalfont, England) was used as a standard.

(v) Glycogen and trehalose contents of cells. Samples of 45 ml were mechanically extracted as described previously (27), with the exception that the biomass pellet was resuspended in 1.25 ml of 0.2 M citrate buffer (pH 4.8) instead of potassium acetate buffer. Glycogen was enzymatically hydrolyzed with 3.0 U of amyloglycosidase (Boehringer) per ml in 0.2 M citrate buffer (pH 4.8), and trehalose was hydrolyzed with 0.25 U of trehalase (Sigma Chemical Company, St. Louis, Mo.) per ml in 0.2 M citrate buffer (pH 5.7) in separate assays overnight at 37°C. The released glucose was measured with an enzymatic kit (Boehringer).

(vi) Elemental composition of cells. The elemental composition of freeze-dried cells was analyzed as described by Gurakan et al. (9).

(vii) Amino acid composition of cells. Samples of 15 mg of freeze-dried cells (9) were hydrolyzed with 5.0 µmol of norleucine as an internal standard in 5 ml of 6 M HCl containing 1 g of phenol per liter for 24 h at 110°C in thoroughly evacuated and sealed glass tubes. Following centrifugation, samples of the supernatant (500 µl) were evaporated with a rotatory evaporator (in vacuo). The residue was dissolved in 2 ml of system buffer (0.20 M sodium, 0.067 M citrate),

TABLE 2. Precursors and amounts of NAD⁺ and NADP⁺ used for synthesis of amino acids and end products made from glucose and ammonium salt^a

Amino acid or product		Precursor(s) ^b	mol/mol of amino acid or product			
			NAD ⁺	NADP ⁺	NAD ⁺ or NADP ⁺	Total produced NADH
Amino acids						
Alanine	Pyr	1	0	0	1	
Arginine	α-Keto	4	−3	1	4–5	
Aspartate-asparagine	Oxal	1	−1	0	1	
Cysteine	3P-Glyc, S ^o /Oxal, AcCoA, S ^o , Ser ^c	2/5 ^c	−3/−4 ^c	0/−1 ^c	2–5	
Glutamate-glutamine	α-Keto	3	−1	1	3–4	
Glycine	3P-Glyc	2	−1	0	2	
Histidine	PrPP	2	1	0	2	
Isoleucine	Pyr, Thr	2	−4	−1	1–2	
Leucine	2 Pyr, AcCoA	5	−2	0	5	
Lysine	α-Keto, AcCoA	6	−3	1	6–7	
Methionine	3P-Glyc, S ^o , Oxal, AcCoA/Oxal, AcCoA, S ^o ^c	4/2 ^c	−6/−5 ^c	−1/−1 ^c	1–4	
Phenylalanine	Ery4-P, 2 PEP	2	−2/0 ^c	0 ^c	2	
Proline	α-Keto	3	−1	−2/1 ^c	1–4	
Serine	3P-Glyc	2	−1	0	2	
Threonine	Oxal	1	−2	−1	0–1	
Tryptophan	Ery4-P, PEP, PrPP, Ser	3	0/2 ^c	0	3	
Tyrosine	Ery4-P, 2 PEP	3	−2/0 ^c	0	3	
Valine	2 Pyr	2	−1	0	2	
Products						
Acetic acid		1	0	1	1–2	
Pyruvic acid		1	0	0	1	
Succinic acid		4	0	1	4–5	
α-Ketoglutarate		3	0	1	3–4	

^a Derived from data published by Jones and Fink (14) and Gancedo and Serrano (8).

^b 3P-Glyc, 3P-glycerate; AcCoA, acetyl coenzyme A; Ery4-P, erythrose 4-phosphate; α-keto, α-ketoglutarate; Oxal, oxaloacetate; PEP, phosphoenolpyruvate; PrPP, phosphoribosylpyrophosphate; Pyr, pyruvate; S^o, sulfur-transferring compound; Ser, serine; Thr, threonine.

^c Depends on the synthetic pathway.

quantities. The amount of glycerol formed can therefore be calculated from the NADH formation accompanying amino acid, extracellular metabolite, and RNA synthesis. The amount of RNA in *S. cerevisiae* (7%), corresponding to 0.69 mmol of NADH produced per g of biomass, was taken from reference 33, while the amount of biomass produced and the protein content of the cells were experimentally determined.

α-Ketoglutarate was assumed to be formed solely from glutamic acid when glutamic acid was used as the nitrogen source. This assumption was made since glutamic acid is used for transamination in most of the biosynthetic pathways of amino acids, resulting in the formation of a large amount of α-ketoglutarate. Transamination was assumed not to be needed in the synthesis of amino acids belonging to the glutamate family (glutamic acid, glutamine, proline, arginine, and lysine), except for arginine and lysine, which contain more than one nitrogen atom. Glutamine was assumed to contribute negligibly to the transaminations. From the experimentally determined amino acid composition of the biomass, it was calculated that 62 mmol of glutamate per mol of glucose should be used in the transamination reactions. This corresponds well to the experimentally determined α-ketoglutarate yield (see Table 3), indicating that the α-ketoglutarate formed most likely arises from transamination processes.

When a mixture of amino acids was used as the nitrogen source, the amounts of NADH formed were calculated from the required net synthesis of the different amino acids. It was assumed that the amino acids were directly incorporated into biomass, if taken up in smaller quantities than the requirements, as determined from the amino acid composition of the biomass (see Table 5). If an amino acid was taken up in larger amounts, the surplus uptake was assumed to be used as a precursor in the relevant amino acid family.

RESULTS

Growth characteristics. The shortest cultivation time was obtained for growth on a mixture of amino acids (Fig. 2). When the glucose was depleted, growth ceased, and thus both heat and carbon dioxide evolution rates decreased to a minimum basic level. The uptake of substrate and formation of ethanol, glycerol, and acetate increased exponentially with time and were directly growth related. The calculated maximal specific ethanol production rates were 1.36 ± 0.12 g of ethanol

per g of dry biomass per h for ammonium, 1.35 ± 0.21 g g⁻¹ h⁻¹ for glutamic acid, and 1.49 ± 0.04 g g⁻¹ h⁻¹ for the mixture of amino acids (the maximal deviations obtained are given). The higher rate of conversion of glucose (Fig. 2) in the cultures with the mixture of amino acids was also reflected in the maximum specific growth rate (Table 3), where the average μ_{\max} of 0.52 h⁻¹ was higher than that for ammonium (μ_{\max} of 0.45 h⁻¹)- and glutamic acid (μ_{\max} of 0.33 h⁻¹)-grown cells (note that the lag phase for ammonium-grown cells was longer than that for glutamic acid-grown cells). Calculations of the specific growth rate from heat measurements gave similar results (Table 3).

Formation of biomass and extracellular metabolites. The choice of nitrogen source affected the formation of many metabolites (Table 3). With glutamic acid as the nitrogen source, the yields of acetic acid, succinic acid, α-ketoglutaric acid, and fumaric acid were higher than those with the other nitrogen sources. The ethanol yield was 8% higher and the glycerol yield was 19% lower than when ammonium was used as the nitrogen source. For cultures grown on the mixture of amino acids as the nitrogen source, the yields of ethanol (14%), acetic acid, and α-ketoglutaric acid were significantly higher and the glycerol yield was decreased by 50% compared with ammonium-grown cultures. The apparent biomass yield on glucose was 1.5 times higher with the mixture of amino acids than it was with either glutamic acid and ammonium as the nitrogen source (Table 3). However, if the biomass was corrected for the amount of carbon taken up in the form of amino acids, a biomass yield of 0.51 C-mol/mol of glucose was attained (Table 3, footnote c), compared with 0.61 C-mol/mol of glucose in the case of ammonium (1 C-mol is the amount of a compound containing 1 mol [12 g] of carbon). No formation of malic acid

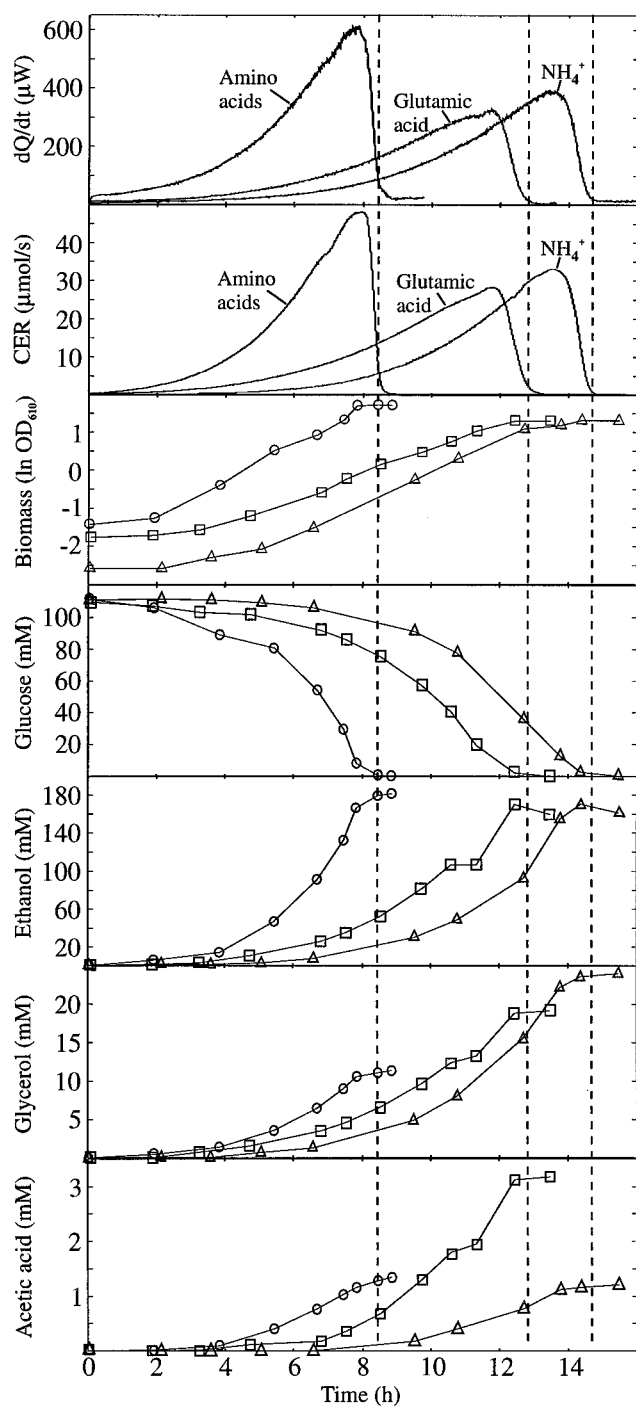


FIG. 2. Representative profiles of anaerobic batch growth and metabolism of *S. cerevisiae* with three different nitrogen sources (ammonium [Δ], glutamic acid [\square], and amino acids [\circ]). Shown are the heat production rate (dQ/dt), carbon dioxide evolution rate (CER), culture turbidity (biomass), and glucose, ethanol, glycerol, and acetic acid concentrations versus time (h). OD_{610} , optical density at 610 nm. The dashed line shows the time when glucose was depleted.

(<0.3 mmol/mol of glucose), *cis*-aconitic acid (<0.0004 mmol/mol), citric acid (<0.05 mmol/mol), isocitric acid (<0.05 mmol/mol), or 2,3-butanediol (<0.6 mmol/mol) could be detected in any of the cultivations. For all nitrogen sources, the uptake of nitrogen per amount of biomass was similar. This is also seen

in the similar elemental composition of the yeast (Table 4). The yield of carbon dioxide (moles per mole of glucose consumed) is higher for the mixture of amino acids than for the other nitrogen sources. However, during growth with either the mixture of amino acids or glutamic acid as the nitrogen source, an additional carbon source(s) is available. Especially in the case of a mixture of amino acids, this resulted in a lower requirement for amino acid synthesis and a higher biomass yield per mole of glucose consumed. Consequently, the yields of both carbon dioxide and heat per amount of biomass formed are lower with an amino acid mixture as the nitrogen source.

The highest biomass yield on ATP (Y_{ATP}) was attained for cultures with the mixture of amino acids as the nitrogen source, while the Y_{ATP} was lower for the ammonium and glutamic acid cultures. The high Y_{ATP} for amino acid cultures is partly due to the fact that the cells can directly incorporate amino acids from the medium and thus will need less ATP to produce biomass. In contrast, the low Y_{ATP} for glutamic acid-grown cells is not expected if the possibility of direct incorporation of glutamic acid in protein synthesis is considered. This indicates an increased energy demand in this case, which may also be reflected by the low specific growth rate.

The carbon and energy balances all closed satisfactorily (Table 3), except for the energy balance of glutamic acid-grown cells. The molar weights of the biomass used in the balance calculations are shown in Table 4. By including corrections for evaporated ethanol, the balances were recalculated. The adjusted values (the maximal deviations obtained are given) for carbon balances were $96.3 \pm 2.9\%$, $96.9 \pm 5.0\%$, and $97.1 \pm 1.5\%$ for ammonium, glutamic acid, and the mixture of amino acids, respectively, and those for energy balances were $99.7 \pm 4.2\%$, $92.2 \pm 7.6\%$, and $98.0 \pm 1.3\%$, respectively. The balances may indicate the formation of some other products, especially in the case of glutamic acid. However, no other products could be detected, despite a search for a large number of substances, including most of the tricarboxylic acid cycle intermediates.

Biomass composition. The results show that the nitrogen sources used have only a small influence on the cellular composition. At the time of glucose depletion, the total protein content of the biomass was similar regardless of the source of nitrogen used (Table 4). The same held for the trehalose content and the unit carbon formula of the biomass, derived from the elemental analysis (Table 4). Ammonium-grown cells attained the highest glycogen content. However, if the standard deviation of the analyses is taken into account, there is no significant difference between the cultures. Overall, the levels of storage carbohydrates were low for cells in the different cultures. The amino acid composition of the biomass was similar for growth on the three different nitrogen sources, except for the content of glutamic acid-glutamine, which was considerably higher in glutamic acid-grown cells, and for smaller variations in the contents of alanine and arginine (Table 5). The alanine content was highest for ammonium-grown cells and lowest for glutamic acid-grown cells, whereas the content of arginine was 60% higher for amino acid-grown cells than for cells grown with the other nitrogen sources.

Amino acid consumption during growth on a mixture of amino acids as the nitrogen source. The consumptions of different amino acids in the mixture were very different (Table 6 and Fig. 3). A large consumption of glutamic acid, aspartic acid, and asparagine, all of which are normally considered to be rich nitrogen sources, was observed. Probably these amino acids can be considered to be additional preferred carbon sources for the cells. These rich nitrogen sources have a large influence on the uptake of other amino acids. The general

TABLE 3. Growth rates, nitrogen uptake, product yields, Y_{ATP} , and balances for anaerobic growth of *S. cerevisiae* on glucose with three different nitrogen sources^a

Parameter and unit	Value with the following nitrogen source:		
	Ammonium	Glutamic acid	Mixture of amino acids
Specific growth rate (h^{-1})			
From OD ₆₁₀	0.45 ± 0.04 ^b	0.33 ± 0.01 ^b	0.52 ± 0.04 ^b
From heat ^c	0.41 ± 0.05	0.35 ± 0.10	0.51 ± 0.05
Nitrogen uptake			
N-mol/mol of glucose	0.12 ± 0.04	0.13 ± 0.04	0.18 ± 0.02
N-mmol/g (dry wt) of biomass	7.3 ± 2.8	7.7 ± 2.2	7.6 ± 0.9
Biomass (C-mol/mol of glucose)	0.61 ± 0.05	0.62 ± 0.03 ^d	0.92 ± 0.06 ^d
Carbon dioxide			
mol/mol of glucose	1.48 ± 0.05	1.53 ± 0.06	1.75 ± 0.09
mmol/g (dry wt) of biomass	89 ± 2	94 ± 3	70 ± 8
Heat			
kJ/mol of glucose	108 ± 4	98 ± 7	112 ± 14
kJ/g (dry wt)	6.5 ± 0.5	6.0 ± 0.4	4.5 ± 0.5
Ethanol			
mol/mol of glucose	1.43 ± 0.03	1.54 ± 0.11	1.63 ± 0.02
mmol/g (dry wt) of biomass	88 ± 5	94 ± 6	66 ± 5
Glycerol			
mol/mol of glucose	0.21 ± 0.02	0.17 ± 0.02	0.10 ± 0.01
mmol/g (dry wt) of biomass	13 ± 2	10 ± 2	4 ± 1
Acetic acid (mmol/mol of glucose)	9 ± 4	25 ± 6	14 ± 2
Pyruvic acid (mmol/mol glucose)	5 ± 1	5 ± 3	3 ± 1
Succinic acid (mmol/mol glucose)	3 ± 2	22 ± 7	5 ± 1
α-Ketoglutaric acid (mmol/mol glucose)	0.5 ± 0.0	64 ± 12	1 ± 0
Fumaric acid (mmol/mol glucose)	<0.08	0.3 ± 0.1	<0.08
Carbon recovery (%)	93.5 ± 3.1	94.1 ± 4.1	95.2 ± 0.8
Energy recovery (%)	96.0 ± 2.9	88.9 ± 6.3	95.3 ± 1.6
Y_{ATP} (g [dry wt] of biomass/mol of ATP)	13.4 ± 0.7	11.9 ± 0.9	16.3 ± 1.3

^a Except as noted, all values are expressed as means derived from three independent growth experiments and maximal deviations. One C-mol is the amount of an (organic) compound containing 1 mol (i.e., 12 g) of carbon. OD₆₁₀, optical density at 610 nm.

^b Mean and deviation from two experiments.

^c Heat production rate.

^d Biomass yields corrected for the carbon taken up directly as amino acids became 0.51 C-mol/mol of glucose when either glutamic acid or the mixture of amino acids was used as a nitrogen source. The corrections were made as follows. For glutamic acid all amino acids belonging to the glutamate family were considered to be derived directly from glutamic acid, and the weight of these amino acids (excluding the weight of water released in the polymerization reactions and the weight of nitrogen) was subtracted from the weight of biomass. For the cultivations with amino acids, the weight of the amino acids taken up (excluding the weight of water released in the polymerization reactions and the weight of nitrogen) was subtracted from the weight of biomass.

amino acid permease GAP1 and the specific proline permease PUT4 are nitrogen catabolite repressed (37) by these rich nitrogen sources and also by ammonium. This repression is seen in the low uptake of valine and phenylalanine, which are taken up by the cell only through GAP1 (Fig. 3B). The repression of PUT4 cannot be interpreted from these data, since the yeast is unable to catabolize proline under anaerobic conditions (6, 13), which is reflected by the absence of proline uptake (Fig. 3B). Methionine, lysine, and histidine were all depleted (Fig. 3A).

Comparison between theoretically calculated and experimentally obtained glycerol formation. With the experimentally determined amino acid composition of the protein content (Table 5) and the amount of NADH produced for each amino acid synthesized (Table 2), the amount of NADH produced per gram of protein was calculated to be between 17.9 and 21.2 mmol of NADH per g of protein. The adjusted amount of NADH produced for the cultivations with glutamic acid was determined to be 11.3 to 13.4 mmol/g, whereas for the mixture of amino acids it was 5.9 to 7.7 mmol/g. The experimental

TABLE 4. Biomass composition of *S. cerevisiae* grown on three different nitrogen sources

Substance (unit)	Value ^a with the following nitrogen source:		
	Ammonium	Glutamic acid	Amino acid mixture
Total protein (g/g [dry wt])	0.63 ± 0.03 ^b	0.58 ± 0.02	0.59 ± 0.03
Glycogen (mg/g [dry wt])	13.9 ± 6.3 ^b	7.3 ± 2.6	5.0 ± 0.5
Trehalose (mg/g [dry wt])	3.6 ± 0.5 ^b	2.9 ± 0.1	3.6 ± 2.1
Ash (mg/g [dry wt])	88.6 ^c	96.7 ± 8.2	96.2 ± 2.1
Unit carbon formula	CH _{1.62} O _{0.47} N _{0.21} ^c	CH _{1.60} O _{0.45} N _{0.20}	CH _{1.63} O _{0.49} N _{0.21}
C-molar mass (g ^d /C-mol)	24.11 ^c	23.63 ± 0.28	24.43 ± 0.03

^a The maximal deviations are given. The samples were taken shortly after glucose depletion and, except as noted, are averages from three independent experiments.

^b Mean and deviation from two experiments.

^c Single experiment.

^d Dry and ash free.

TABLE 5. Amino acid composition of yeast cells grown on three different nitrogen sources

Amino acid	mg/g of protein ^a with the following nitrogen source:		
	Ammonium	Glutamic acid	Amino acids
Alanine	87.4	60.0 ± 1.0	74.0 ± 3.6
Arginine	60.3	58.7 ± 0.5	94.9 ± 4.3
Asparagine-Aspartic acid	98.6	95.9 ± 1.3	97.0 ± 2.4
Cysteine	13.1	12.7 ± 0.3	10.8 ± 0.5
Glutamine-Glutamic acid	144.7	216.2 ± 0.1	116.8 ± 3.1
Glycine	47.8	47.9 ± 0.1	46.1 ± 2.0
Histidine	22.4	22.1 ± 0.8	22.5 ± 0.6
Isoleucine	49.3	48.8 ± 0.2	47.5 ± 2.0
Leucine	71.8	73.1 ± 0.1	69.6 ± 2.0
Lysine	79.9	78.0 ± 0.6	76.4 ± 3.0
Methionine	16.7	16.5 ± 0.1	15.8 ± 1.2
Phenylalanine	40.3	41.3 ± 0.2	38.5 ± 1.8
Proline	35.1	38.6 ± 0.7	33.5 ± 1.2
Serine	52.0	55.8 ± 0.8	50.6 ± 2.4
Threonine	48.7	50.6 ± 0.8	48.5 ± 1.7
Tryptophan	14.3 ^b	14.3 ^b	14.3 ^b
Tyrosine	33.9	36.0 ± 0.7	32.9 ± 0.9
Valine	60.3	59.5 ± 0.3	59.1 ± 2.1

^a Except as noted, the mean is derived from one, two, and three independent growth experiments for ammonium, glutamic acid-, and amino acid-grown cells, respectively. The maximal deviations are given.

^b Representative value derived from reference 2.

results for biomass yield, protein content of the cells, and yields of extracellular metabolites were used to calculate the total amount of NADH produced. The corresponding theoretically calculated glycerol yields were compared with the experimentally obtained ones (Table 7). The experimental yields of the cultures grown with ammonium or glutamic acid agreed well with the lower-range values of the theoretical yields. The theoretically and experimentally determined yields of the amino acid-grown cultures differed more, but the lower-range value is still reasonably close to the experimentally obtained value.

TABLE 6. Net uptake of amino acids from the medium containing a mixture of amino acids

Amino acid	Net uptake (μmol/liter)	Amt taken up per initial amt (%)
Alanine	638	35
Arginine	938	74
Asparagine	3,697	87
Aspartic acid	2,616	78
Cysteine	NM ^a	NM
Glutamine	52	70
Glutamic acid	2,081	86
Glycine	158	15
Histidine	180	100
Isoleucine	89	59
Leucine	366	73
Lysine	480	100
Methionine	121	100
Phenylalanine	120	43
Proline	0	0
Serine	547	88
Threonine	257	88
Tryptophan	349	14
Tyrosine	51	21
Valine	139	26

^a NM, not measured.

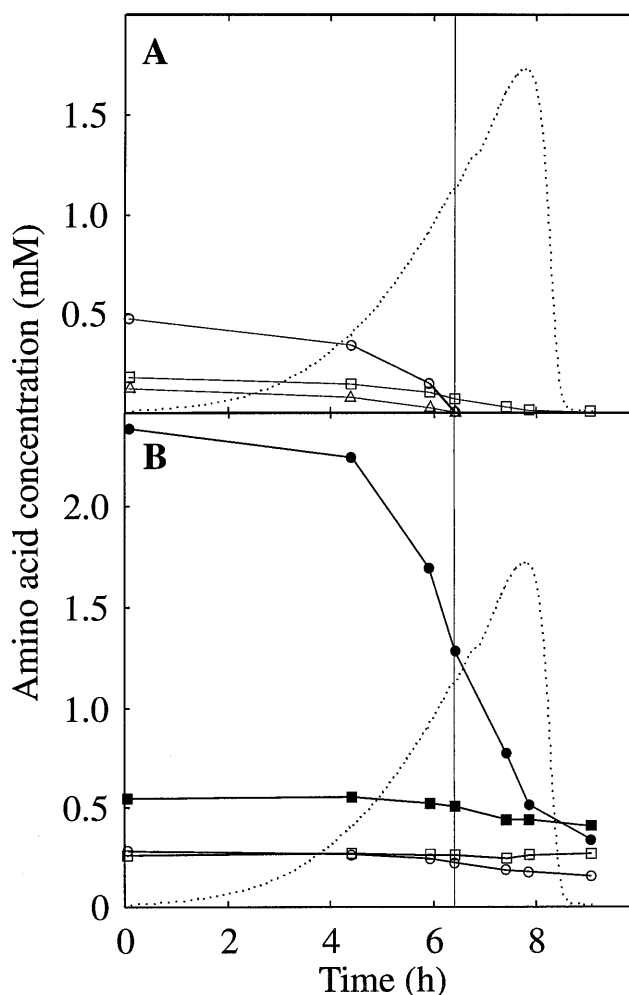


FIG. 3. Amino acid consumption for anaerobic batch growth of *S. cerevisiae* with a mixture of amino acids as the nitrogen source. The carbon dioxide evolution rate (dotted line) is indicated for comparison. (A) Δ , methionine; \square , histidine; \circ , lysine. (B) \bullet , glutamic acid; \blacksquare , valine; \square , proline; \circ , phenylalanine.

DISCUSSION

Enthalpy changes are involved in all reactions in the cell. Consequently, the rate of heat evolution reflects the overall metabolic activity (10, 35). Carbon dioxide, on the other hand, is produced in only some reactions. The formation of glycerol is an example of a reaction which gives rise to an enthalpy change but does not give rise to carbon dioxide. Under anaerobic conditions, the heat and carbon dioxide evolutions give basically the same information, as can be seen in Fig. 2. This is due to the fact that the formation of ethanol is, quantitatively, the major source of both heat and carbon dioxide evolution (10, 35).

The specific rate of growth of our strain on ammonium and glutamic acid (Table 3) is consistent with data from the literature (0.46 h^{-1} [26] and 0.47 h^{-1} [36] for ammonium and 0.38 h^{-1} for glutamic acid [36]). The specific growth rate for cells grown on the mixture of amino acids as the nitrogen source is high (0.52 h^{-1}), which may be explained by the decreased need for amino acid synthesis in this case. The positive effect of amino acids on the growth and fermentation rates has previously been noted by Thomas and Ingledew (30, 31).

Under anaerobic conditions, the Y_{ATP} can be accurately

TABLE 7. Theoretical and experimental yields of glycerol for the three different nitrogen sources

Nitrogen source	Glycerol production (mol/mol of glucose) resulting from:				
	Synthesis of amino acids	Synthesis of metabolites ^a	Synthesis of RNA ^b	Total theoretical calculation	Expt measurement ^c
Ammonium	0.18–0.22	0.03–0.04	0.01	0.22–0.27	0.21 ± 0.02
Glutamic acid	0.12–0.14	0.05–0.08	0.01	0.18–0.23	0.17 ± 0.02
Mixture of amino acids	0.09–0.11	0.04–0.06	0.02	0.15–0.19	0.10 ± 0.01

^a Acetic acid, succinic acid, pyruvic acid, and α -ketoglutarate.^b Calculated from reference 33.^c Means and the maximal deviations derived from three independent growth experiments.

calculated, since only fermentative metabolism has to be accounted for. The obtained biomass yields on ATP were different for the cultures with different nitrogen sources (Table 3). According to Verduyn et al. (34), the amino acid synthesis accounts for only approximately 5% of the total energy requirement, which is not enough to explain the higher Y_{ATP} for the cultures with a mixture of amino acids. For amino acid-grown cells, a possible ATP-demanding process (compared with the case for ammonium-grown cells) may be the uptake of amino acids. One may consider the case of growth on one amino acid, e.g., glutamic acid. The transport of glutamic acid is known to take place by proton symport by usage of two or three protons (11). The transport of ammonium is a uniport system directly driven by the electrochemical plasma membrane potential, generated by the plasma membrane ATPase (23, 32), in which the ion is taken up and the proton is later excreted to keep a constant intracellular pH. This excretion of one proton per ammonium taken up has been shown for *Debaryomyces* and *Candida* spp. (12, 17). Thus, the energy demand for glutamic acid uptake should be two to three times higher than that for ammonium. According to Verduyn et al. (34), the ATP cost of ammonium transport is 16% of the total demand of ATP (corresponding to 11.9 mmol of ATP per g of dry biomass). If it is assumed that all other ATP costs are equal, the Y_{ATP} for glutamic acid-grown cultures can be calculated from the Y_{ATP} for ammonium-grown cultures. The Y_{ATP} for the glutamic acid cultivations, calculated this way, becomes 10.2 to 11.6 g of biomass per mol of ATP, which can be compared with the experimentally obtained value of 11.9 g/mol.

When making the comparison between ammonium- and amino acid-grown cultures, one should, however, take into account the carbon which is supplied in form of amino acids. A biomass yield corrected for this (Table 3, footnote c) was used to recalculate the biomass yields on ATP for the cultures grown with glutamic acid and the mixture of amino acids. Calculated this way, the Y_{ATP} was found to be 9.8 g of biomass per mol of ATP for glutamic acid-grown cells and 9.0 g/mol for amino acid-grown cells. Both of these values are lower than the Y_{ATP} for ammonium-grown cells, which may be explained by the more energy-demanding transport of amino acids.

The study shows that if the synthesis of amino acids can be reduced by using amino acids as a nitrogen source, reducing equivalents will be formed to a lesser degree and, thereby, the glycerol yield will decrease. When glutamic acid was used as the nitrogen source, the product pattern was markedly changed in one particular aspect compared with the case for both ammonium and a mixture of amino acids as nitrogen sources. Glutamic acid is the donor of nitrogen in many of the biosynthetic pathways of amino acids. Consequently, an excess of α -ketoglutarate is to be expected in glutamic acid-grown cultures, which also has been noticed earlier (18). Further

conversion of α -ketoglutarate in the tricarboxylic acid cycle is indicated by the increased amounts of succinic and fumaric acids. The recovery of glutamic acid was calculated to be 92 (± 11)% (the maximal deviation obtained is given). In this calculation it is assumed that the glutamic acid taken up from the medium gives rise to all extracellular α -ketoglutaric acid, succinic acid, and fumaric acid, as well as to amino acids in the biomass belonging to the glutamate family (glutamic acid, glutamine, proline, arginine, and lysine). This shows that the assumptions concerning the utilization of glutamic acid are reasonable. The reduced synthesis of α -ketoglutarate from glucose causes less NADH to be formed, which reduces the glycerol yield. When a mixture of amino acids is used as the nitrogen source, the glycerol yield is even further reduced and the ethanol yield is increased.

The comparison between the theoretical yields of glycerol, based on NADH calculations, and the yields obtained experimentally (Table 7) shows that, for all nitrogen sources studied, the lowest values of the theoretical yields are in good agreement with the experimental ones. The calculated value of NADH produced (17.9 to 21.2 mmol/g of protein) with ammonium as the nitrogen source agrees with values found in the literature (22.6 mmol/g [15], 22.0 mmol/g [33], and 21.9 mmol/g [26]). Differences between theoretical and experimental values for NADH, and hence glycerol, formation can be found in the literature (33) and may be due to uncertainties about the biosynthetic pathways, which lead to different results in the calculations of the amounts of NADH produced per gram of protein. Experimental errors in the determinations of the protein and amino acid contents of the biomass will also add to the uncertainties. However, the theoretical range of the glycerol yield is caused not only by uncertainties about the metabolic pathways but also by the different possible choices of cofactors in several oxidation or reduction steps. The agreement between the experimental glycerol yield and the lower-range yield of the theoretical calculations indicates that the metabolism is regulated to minimize NADH formation.

In conclusion, the results show that product formation by *S. cerevisiae* under anaerobic conditions is affected by the nitrogen source. Of particular practical relevance is the fact that if amino acids are added to the medium, the yield of glycerol is reduced and the yield of ethanol is increased. Although the product formation changed, no significant changes in the biomass composition in terms of carbohydrate storage, protein content, or protein composition were found when different nitrogen sources were used.

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